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PERSISTENT COLIFORM CONTAMINATION IN LECHUGUILLA CAVE POOLS

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Despite designated trails, limited access, water pitchers, and other low-impact caving techniques, coliforms, a bacterial indicator of fecal contamination, are found in the drinking-water pools of Lechuguilla Cave, Carlsbad Caverns National Park, New Mexico. Researchers, concerned cavers, and Carlsbad Caverns National Park Service staff have restored contaminated areas; nevertheless, coliforms persist over time. Investigation of the problem showed that water-siphoning tubing supports strong biofilm growth in the same pools in which coliforms are present, suggesting that the biofilm is a factor in coliform persistence. We took a three-pronged approach in exploring this problem: 1) Identification of coliform presence and persistence using +/- coliform indicator quantification tests, 2) Culturing of coliforms in the presence and absence of biofilm to test whether the biofilm enhances coliform growth, and 3) Assessment of biofilm growth on tubing by suspending tubing of varying chemical compositions in cave water. Results indicated that coliform levels exceed those set by the Environmental Protection Agency for drinking-water. Additionally, coliform populations increased in the presence of the biofilm. VWR Tygon showed the heaviest biofilm development while silicone and Teflon tubing did not support any visible biofilm growth in lab experiments. Remediation efforts and management recommendations for the current problem are discussed.

Lechuguilla Cave, located 5.6 km WNW of Carlsbad Cavern in Carlsbad Caverns National Park, Eddy County, NM, contains numerous pools with unique microbial communities that have been subjected to impact by human visitors (Mallory *et al.* 1995, Northup *et al.* 1997). The discovery in 1986 of Lechuguilla's extensive passages beyond the entrance area provided scientists a spectacular cave in which to study speleogenesis, unusual speleothems, and geomicrobiological interactions (Boston *et al.* 2001, Provencio & Polyak 2001, Cunningham *et al.* 1995, Dawson 1996, Hill 2000, Northup *et al.* 2000, Palmer & Palmer 2000, Polyak & Provencio 2000, Turin & Plummer 2000). As the deepest cave in the continental United States, with a total surveyed length of 170 km and a depth of 475 m (Turin & Plummer 2000), Lechuguilla also showed potential as an analogous environment for extraterrestrial life (Boston 2000).

Lechuguilla's pristine nature and numerous possibilities for science and discovery have encouraged various conservation measures including the establishment of camps, designated trails, urine dumps, and drinking sources, and limiting of organic carbon enrichment (Northup *et al.* 1992). Despite preservation efforts, human contact with the ground waters has led to unintentional contamination of Lechuguilla's sources of drinking-water, posing an unusual importance due to the limited access (Boston 1999, Northup *et al.* 1997, Northup *et al.* 2000; Fig.1).

Figure 1. Red Lake study area, located in the Western Branch of Lechuguilla Cave, has been closed due to coliform contamination in the water source. Photo by Val Hildreth-Werker.

COLIFORM PRESENCE AND PERSISTENCE

Coliforms within Lechuguilla were first discovered in 1995 within urine disposal areas and nearby trails in one case (Northup *et al.* 1997). Subsequent studies also found positive coliform results in several soils and drinking source locations, revealing a notable problem (Boston 1999). Coliforms are

Figure 2. Biofilms from siphoning hoses, found in cavers' water bottles. Photo by Val Hildreth-Werker.

important indicator organisms for potential pathogens responsible for waterborne diseases. Waterborne diseases arise when pathogens living in water are transmitted through ingestion or contact with water (Shagam *et al.* 2000), although some can enter through the skin (Chapra 1996). Short-term effects such as fever, vomiting, bloody diarrhea, cramps, nausea, headaches, fatigue, jaundice, and in some cases, kidney failure can appear if pathogenic organisms are present. Coliforms may not just be indicators, but like enteropathogenic *E. coli*, can also be serious pathogens themselves (Todar 2002). Originating only from the intestines of warm-blooded animals (Chapra 1996), coliform presence within Lechuguilla Cave is related to either human introduction or surface infiltration (Turin & Plummer 2000).

Several studies of pool chemistry in Lechuguilla Cave have found low total organic carbon/dissolved organic carbon levels, establishing the oligotrophic, low-nutrient nature of Lechuguilla pools (Dawson 1996, Northup *et al.* 1992, Turin and Plummer 2000). Given the natural low carbon/nutrient availability and isolation for long periods of time from perceived high nutrient sources (i.e. human contact), persistence of non-native, high nutrient-requiring organisms such as coliforms was hypothesized to be limited. However, coliforms are still present in Lechuguilla's pools.

BIOFILM ENHANCEMENT OF COLIFORM GROWTH

Slime-like biofilms present within siphon hoses in Lechuguilla water sources may act as a potential carbon/energy source for introduced coliform bacteria. When a liquid and a surface come into contact, bacteria present within that liquid are attracted and adhere to the surface forming a glycocalyx or carbohydrate coat bonded to proteins and lipids (Lappin-Scott & Costerton 1997). Microcolonies then form and organic and inorganic matter are trapped within the glycocalyx where nutrients can become very concentrated (Lappin-Scott & Costerton 1997). The microbial biofilm can act as a nutrient reservoir, increasing the chances of survival for potential pathogens, causing considerable concern within the drinking-water and food industries (Lappin-Scott & Costerton 1997).

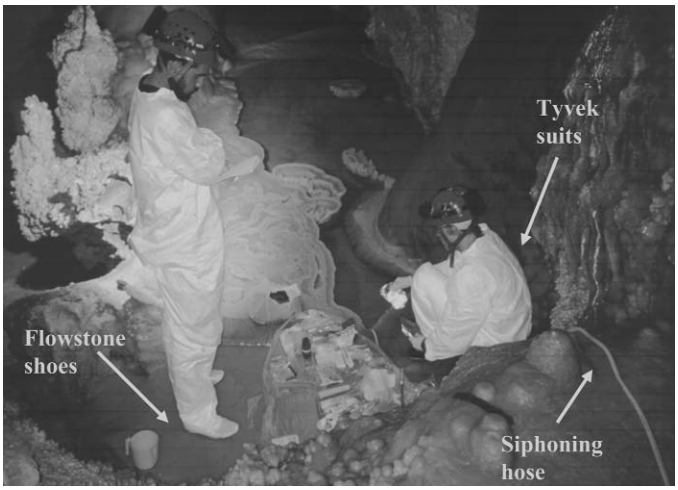

Figure 3. Biofilm sampled from water siphoning hose at Red Lakes Pool located in the Western Branch of Lechuguilla Cave. This figure also demonstrates flowstone shoes, Tyvek suits, and other sterile techniques used during sampling. Andy DuFrane left, Andrea Hunter right. Photo by Val Hildreth-Werker.

Cavers using Lechuguilla water sources have also found the threat of disease to be a concern when large amounts of biofilm are found floating in their water bottles (Fig. 2).

Lechuguilla Cave pools contain dimorphic prosthecate bacteria (Boston 1999, Northup *et al.* 1992), morphologically similar to *Hyphomicrobium* and *Caulobacter* spp. (Poindexter 1992), as well as *Escherichia coli* introduced by humans (Boston 1999, Northup *et al.* 1997). The nutrient requirements and metabolism of these two microbial communities may act in a synergistic (non-obligatory) relationship. Although *Hyphomicrobium* and *Caulobacter* spp. are indigenous to many pools in Lechuguilla Cave, the introduction of water hoses into the cave has provided a place for these species to form biofilm communities and thrive (Boston 1999; Fig. 3). The persistence of coliforms in pools with biofilm-covered tubing has led to the speculation that the biofilm produced on the tubing may serve as a carbon/energy source aiding the persistence of coliforms.

BIOFILM GROWTH ON TUBING

Some types of tubing contain chemicals that leak low-molecular-weight carbon compounds (C1, C2, C3) over time after submersion in water (Budde 1995). These types of tubing may provide a carbon source for oligotrophic microbes like *Hyphomicrobium* and its relatives that are able to scavenge very low quantities of organic compounds in solution (Hirsch and Conti 1964a, b, Whittenbury *et al.* 1970, Sperl and Hoare 1971, DeBont *et al.* 1981, Suylen *et al.* 1986).

VWR brand Tygon tubing was initially installed in Lechuguilla as a siphoning hose in both Deep Secrets and Red Lake, while the Big Sky water source had vinyl tubing (Figs.

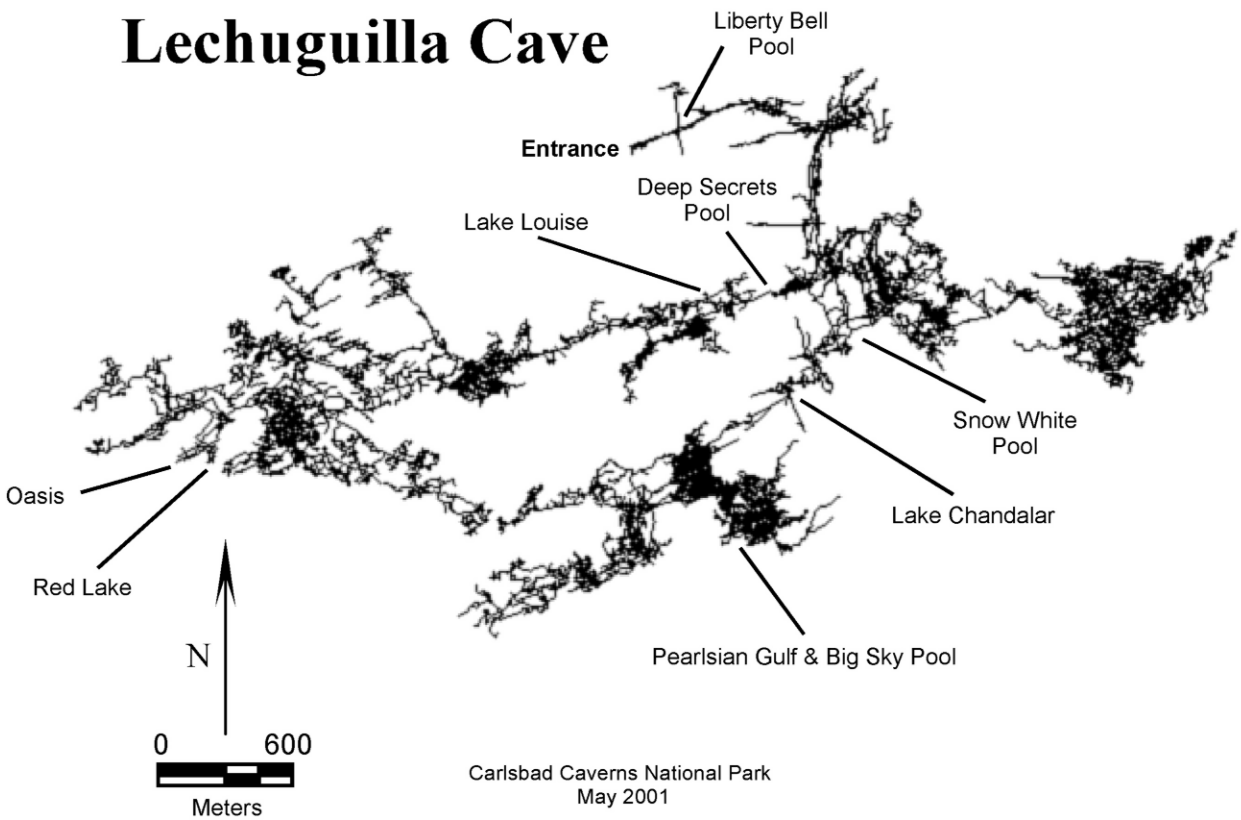

Figure 4. Plan view map of Lechuguilla Cave and study sites. Map by Stan Allison, Carlsbad Caverns National Park, May 2001.

3 & 4). These types of tubing were installed for both convenience and conservation. Some of the pools are located in awkward places, so using a siphoning hose to access water has proven to be both beneficial and convenient for cavers. The placement of hoses also helped to conserve areas surrounding the pools since direct pool access was no longer necessary. However, these hoses may be poor choices as they may act as nutrient sources for large amounts of microbial biofilm. We hypothesized that native organisms (e.g. dimorphic prosthecate bacteria such as *Hyphomicrobium* spp.) prefer the use of low-molecular-weight carbon compounds leaking from siphoning hoses and their formation of high-molecular-weight carbon compound biofilms serve as an energy source for introduced coliforms that prefer complex carbon compounds as nutrient sources. The presence of siphoning hoses inflates native organism populations, which in turn may prevent rapid die-off of coliforms and lead to coliform growth.

METHODS

FIELD TECHNIQUES

Coliform presence was tested at Liberty Bell Pool, Snow White Pool, Deep Secrets Pool, Lake Louise, Red Lake, and Oasis Pool in Lechuguilla Cave (Figs. 3 & 4). Pools that contained siphoning hoses, water pitchers or no water-aiding devices at all (differences noted in Tables 1 and 2) were tested for the presence or absence of total coliforms using LaMott Company coliform indicator test kits model TC-5 (Table 1). Five individual vial tests plus a control were used at each site. Ten mL of pool water were aseptically poured into each media vial and left motionless for 48 hours. If the media became orange-yellow with bubbles, the test was considered positive for total coliforms; a red was indicative of a negative test.

Coliforms were quantified from Deep Secrets Pool, Lake Louise, Red Lake, Pearlsian Gulf, Lake Chandalar, and Big Sky Pool (Fig. 4). Standard United States Geological Survey protocols were used to select for total coliforms (Webb *et al.* 1998, Myers & Sylvester 1998). Samples were aseptically collected and brought back to Deep Seas camp within four hours to be aseptically processed using the most probable number method during January 1999 tests (Koch 1994) and the membrane filtration technique during January 2001 tests (Myers & Sylvester 1998). Samples were filtered onto pre-made media plates that select for total coliforms and were then placed into an on-site incubator at 35°C for 24 hours. Colonies that were red, round, raised and smooth with a golden-green metallic sheen were considered positive and counted.

Biofilm samples were cultured and collected from pools containing siphoning hoses (equally coated with biofilm both in and out of the water), including Deep Secrets and Red Lake located in the Western Borehole and Big Sky Pool in the Southwest Branch (Fig. 4). It was determined from Boston's 1999 experiments that *Hyphomicrobium* spp.-like organisms isolated from Deep Secrets pool showed a nutrient preference towards low quantities of organic compounds in solution, such as C1, C2, and C3 carbon compounds (Boston, personal communication, 2000). For this purpose, the Atlas protocol for *Hyphomicrobium* medium (specific to urea and methanol) was

Table 1. Coliform +/- results from Lechuguilla pool drinking sources (# positive/# total tests).

Date	Red Lake[b]	Lake Louise[a,c]	Deep Secrets[a,b]	Liberty Bell	Snow Wt. Passage	Oasis[c]
1/15/1999 (P.Boston)	(3/8) (Sm.Pools) ND (Lg.Pool)	ND	ND	ND	ND	ND
1/15/2000 (A.Hunter)	(1/5) Lg.Pool	(4/5)	(0/5) Lg.Pool	ND	ND	ND
11/18/2000 (A. Hunter)	(0/5) Lg.Pool	(4/5)	(1/7) Sm.Pool (3/5) Lg. Pool	ND	ND	ND
1/26/2001 (A. Hunter)	(2/4) Lg. Pool	(4/6)	(4/6) Lg. Pool	(0/5)	(0/5)	(1/5)

[a] Denotes current drinking water source. Others have been used in the past during early exploration and rescue situations or have been closed for research purposes or contamination.

[b] Represents pools with water siphoning hoses during 2001.

[c] Represents pools with water pitchers or dipping cups during 2001.

prepared for organism collection within Lechuguilla's pools during 2000 and 2001. Using aseptic techniques, 1 ml inoculating loops were used to scrape off one loop-full of tubing biofilm cells to inoculate the solid medium in vacuum vials. Sterile syringes were then placed into the vacuum vial and excess air was extracted, creating a near anoxic environment. An additional 5 mL of visible biofilm from tubing was collected using a sterile syringe. The biofilm was then placed into a sterile vial containing pool water, transported out of the cave and stored in the lab refrigerator for use in laboratory experiments.

Water was collected aseptically in sterile bottles from Deep Secrets and Lake Louise for use in laboratory experiments. Water samples were stored at 20°C during transport to the lab where they were immediately used in the tubing experiment. Aseptic or clean techniques, including the use of Tyvek suits, sterile gloves, flowstone shoes (clean, rubber-soled slippers) and anti-bacterial wipes, were used at all sites while collecting samples at pool ledges to minimize contamination (Fig. 3). Water samples from Deep Secrets were not collected directly near the siphoning hose.

LABORATORY TECHNIQUES

Total coliform medium was prepared using protocols from the United States Geological Society (Myers and Sylvester 1998). The medium, containing 4.8 grams of m ENDO broth MF, 1.5 grams of Bacto agar and 100 mL of 2% ethanol solution, was boiled at 95–96°C then cooled, poured aseptically into 50 mm petri dishes, and stored in the refrigerator.

Coliform growth preference was tested by counting the number of coliform colonies grown in the presence and absence of biofilm over time. Plates containing LB medium plus ampicillin were used to grow ampicillin-resistant *E. coli* (Sambrook *et al.* 1989). LB medium was prepared by adding bacto-tryptone, bacto-yeast extract, sodium chloride and Bacto agar to double-distilled water (Sambrook *et al.* 1989). One ml of full-strength ampicillin was added to every one mL of LB solution. It was necessary to use ampicillin-resistant *E. coli* so that other organisms not resistant to ampicillin would be killed and would not grow on the LB media plates. Three sets of vials containing seven vials each (one for each day) were prepared as follows. One set of vials containing 20 mL of *Hyphomicrobium* medium (American Public Health Association 1989, Atlas 1995) plus 0.2 mL of glucose (giving a 1% glucose solution) with one loop of ampicillin-resistant *E. coli* (1 inoculating loop = approximately 1,000,000 cells) served as the control (Fig. 5). The second set of vials served as the culture experiment containing 18 mL of *Hyphomicrobium* medium plus a 1% glucose solution, 2 mL of *Hyphomicrobium* culture, and one loop of ampicillin-resistant *E. coli* (Fig. 6). The third set of vials served as the biofilm experiment containing 20 mL of *Hyphomicrobium* medium plus glucose, one loop of slime from cave tubing and one loop of ampicillin-resistant coliforms (Fig. 5). All vials contained an equal number of ampicillin-resistant *E. coli* cells. The vials were placed on a shaker while incubating at 20°C for 15 minutes. Serial dilutions using sterile cave water were then performed giving a 10^{-6} dilution. After 24-hours of incubation, 0.1 mL from the 'day 1' vials in each 10^{-6} dilution set was spread onto triplicate LB plates, creating nine new plates per day. All plates were quantified at 0 CFU on Day 0 (Fig. 6). The plates were then incubated for 24-hours at 30°C. Coliform colonies grown on plates were counted after each 24-hour period and plates were discarded. This procedure was repeated daily for one week plating 'day 2' vials on day 2, etc., each day representing its respective vial and corresponding amount of incubation time (Fig. 6).

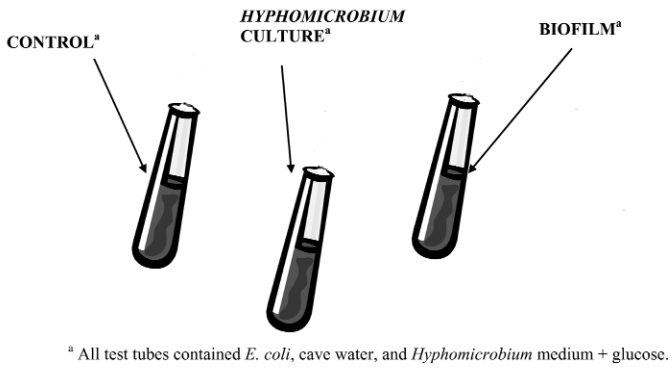

Figure 5. Contents of original dilution tubes used in coliform growth with/without biofilm experiment.

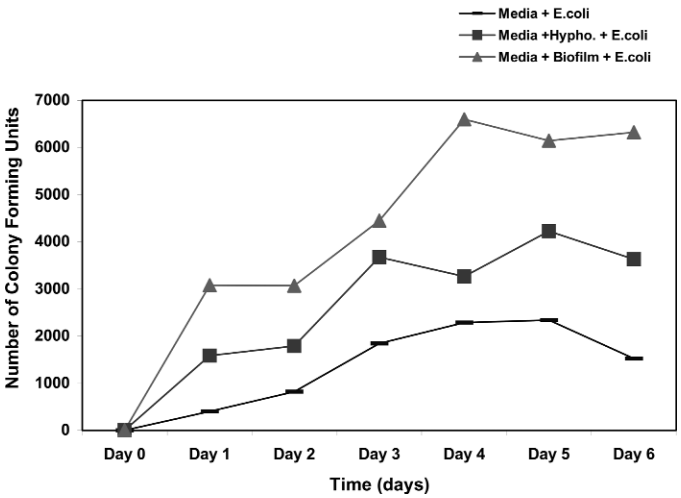

Figure 6. *E. coli* growth in the presence of *Hyphomicrobium* culture and biofilm from 1/100,000 dilutions of original cultures. 1) Cave Water and Media 2) Cave Water, Media, and *Hyphomicrobium*-Culture, and 3) Cave Water, Media, and Biofilm.

Microbial preference for growth on certain types of tubing was determined by suspending a variety of hoses with differing chemical compositions and amounts of organics in water taken from cave pools. Tubing types included in the experiment were natural rubber latex (Cole-Parmer Catalog number P-06402-10); silicone (platinum-cured) Masterflex (Cole-Parmer Catalog number 96410-15); Norton Tygon R-1000; Teflon (FEP, fluorinated ethylene propylene; Cole Parmer Catalog number P-06406-12); Nalgene Premium Tygon 180; and VWR Brand Tygon (Cole-Parmer catalog number 40-80000090). Pieces of tubing were sterilized for 30 minutes in a 121°C autoclave at 10 atm. Tubing was suspended using sterile fishing line in sterile test tubes containing 40 mL of unsterilized cave water with native microbial communities for five months using sterile fishing line. Vials with tubing were covered in tin foil and placed in a dark incubator at 20°C to replicate cave temperature and darkness. The tubing was examined for signs of visible growth after 6, 8 and 20 weeks of incubation.

STATISTICAL ANALYSIS

Colony count data from the *E. coli*/*Hyphomicrobium*/ biofilm experiment were analyzed using the analysis of variance (ANOVA) procedure in the SAS software release 6.12 (SAS Institute, Cary, NC, USA). Data were considered significant at $P < 0.05$.

RESULTS

Presence/absence coliform tests revealed the presence of coliforms in most drinking-water pools in the Western Borehole (Table 1). Red Lake, Lake Louise, and Deep Secrets pools consistently had positive coliform results; Liberty Bell and Snow White Passage remained negative. Pools with siphoning hoses, dipping cups, or water pitchers are noted in Tables 1 and 2. On average, pools with siphoning hoses contained more colony forming units/100 mL than those with dipping cups or water pitchers. Pools that tested positive for coliforms had quantification tests run, determining the level of severity. Coliform counts showed that as of January 2001, the large pool at Red Lake had the highest number of colony forming units (27 organisms/100 mL) of the sites tested (Table 2). Lake Louise had the second highest number (19 organisms/100 mL). All counts taken during January 2001 except for Deep Secrets (reported as ideal colony count) were non-ideal colony counts. A non-ideal colony count is the terminology used by the United States Geological Society to represent less than 20 colonies counted in a 100 mL sample. An ideal colony count represents 20–60 colonies counted in a 100 mL sample (Myers & Sylvester 1998).

A lab experiment testing whether coliforms grew faster in the presence of cave biofilm given the same amount of organic carbon and nutrients showed that coliforms significantly preferred growing with the biofilm ($P=0.0001$; Fig. 6). Coliforms also favored medium containing biofilm over medium containing cultured *Hyphomicrobium*-like organisms (Fig. 6).

Results from the experimental testing of microbial biofilm growth on various tubing over five months were consistent. Silicone (platinum-cured) Masterflex, Norton Tygon, and Teflon (FEP, fluorinated ethylene propylene) showed no visible growth of biofilm at any time during the experiment. Nalgene Premium Tygon 180, and natural rubber latex produced visible moderate biofilms at 6 weeks and remained constant for the remaining 24 weeks. VWR Brand Tygon showed visible heavy biofilm growth at 6 weeks and remained constant throughout the entire five-month period. The experiment showed rapid colonization to reach maximum growth of biofilm in all types of tubing in 6 weeks.

Table 2. Coliform quantification results from Lechuguilla pool drinking sources.

Date	Deep Secrets Pool (CFU/100mL)[a,b]	Lake Louise (CFU/100 mL)[a,c]	Red Lake (CFU/100mL)[a,b]	Pearlsian Gulf (CFU/100 mL)[a,c]	Lake Chandalar (CFU/100mL)[a,c]	Big Sky Pool (CFU/100 mL)[a,b]
1/15/99 (P.Boston)	ND	ND	5400 (Sm. Pool 1) 2200 (Sm. Pool 2) 0 (Lg. Pool)	ND	ND	ND
1/26/01 (A.Hunter)	4	19	27 (Lg. Pool)	3	2	0.4

[a] Denotes abbreviation: CFU: Colony Forming Unit.
[b] Represents pools with water siphoning hoses during 2001.
[c] Represents pools with either a dipping cup or water pitcher during 2001.

DISCUSSION

PRESENCE AND PERSISTENCE OF COLIFORMS

As indicators for fecal contamination and disease-causing bacteria, coliforms are useful representative species for which to test in potentially disturbed environments like the drinking pools in Lechuguilla Cave, New Mexico. Confirmed presence of coliforms in these pools has indicated fecal contamination, and consequent closure of some of the drinking-water sources available to cavers, researchers and explorers. Since the extent of coliform contamination was not recognized until 2001, no pre-2001 data are available for most pools listed in Table 2 with the exception of Red Lake, a prime concern and the only pool tested in 1999. Currently, Lake Louise and Deep Secrets are the only drinking-water sources in the Western Borehole due to other pools either being contaminated or off-limits due to research (Fig. 1). Red Lake, for instance, has been closed for several years due to coliform contamination and Oasis Pool has been secluded for microbiological research (Allison, personal communication, 2001). Pools in Liberty Bell and Snow White Passage were used as drinking-water sources during the 1991 rescue of an injured caver, and not used as microbiologically safe and reliable sources (Allison, personal communication in 2001). Lechuguilla's drinking pools are few and far between and should be regarded as both valuable drinking-water resources and research study sites. If coliform contamination in these environments persists, then the resources may have to be put off limits to avoid negative human health and safety issues. Alternative methods for controlling coliform introduction and amelioration of the techniques employed to obtain drinking-water from cave pools may make such draconian measures unnecessary. However, such management decisions are complex.

In addition to the major concern for the presence of coliforms in Lechguilla's low-nutrient pool environments, there is also a well-dispersed presence of fecal contaminants in the soils of some camps and urine dumps. We presume that cavers and their boots have tracked coliforms from the contaminated soil areas onto the flowstone slopes leading to the pool environments (Fig. 3). This was shown when mud from cavers' boots was found in some of the small pools located on the flowstone slope above the large pool at Red Lake. Currently, the Safe Drinking-Water Act and Environmental Protection Agency (EPA) national primary drinking-water standards for total coliform detection are set at a maximum contaminant level goal of 0 organisms/100 mL (Environmental Protection Agency 1999). Coliform contamination (2,200 to 6,600 organisms/100 mL) in these small pools also exceeds the accepted coliform level for recreating in public waters set by the Environmental Protection Agency. For example, the state of New Mexico has set recreational standards for fecal coliforms at 100 organisms/100 mL for Dry Cimarron River, 200 organisms/100 mL for the San Francisco, San Juan, Canadian, Pecos and Gila River Basins, and 1000 organisms/100 mL for the main corridor of the Rio Grande above American Dam to below Percha Dam (State of New Mexico 1995). Albuquerque's reclaimed water discharge has a maximum daily fecal coliform limit of 200 organisms/100 mL, however, some parts of the Rio Grande allow 2000 organisms/100 mL for one sample (Shagam *et al.* 2000). The Environmental Protection Agency has set criteria for swimming at fewer than 200 organisms/100 mL; 1000 organisms/100 mL for fishing and boating and 2000 organisms/100 mL for domestic water supplies (Boston 1999). Coliform concentrations in Red Lake's small pools (2,200 to 6,600 organisms/100 mL) were higher than all of these standards. While high coliform concentrations were found in Red Lake's small pools in January 1999, the large pool at Red Lake was negative at that time, but two years later it was positive when retested in January 2001. The Red Lake area had been closed to cavers during that time, suggesting that coliforms might have migrated from the heavily contaminated small pools downward into the larger water source traveling in fluid overflow down flowstone slopes.

Other Lechuguilla pools containing much smaller numbers of coliforms also should be viewed with concern. If located on the surface, most of the tested Lechuguilla water sources would be suitable for swimming, fishing or boating. However, only those with negative results would be considered potable sources of water for human consumption. Lechuguilla pools are indispensable in supplying water to visiting humans and

are critical habitats for native microorganisms (Mallory *et al.* 1995). Therefore, addressing the coliform and fecal contamination problem is critical for health, safety, and introduced organics.

Sampling procedures also tested very small volumes of water near the siphoning hoses in pools where siphoning hoses were present. Spatial variance of coliform numbers could therefore be dependent on where coliforms concentrate. If coliform bacteria prefer biofilms and concentrate near them, coliform quantification test results from other parts of the pool will be lower. Regardless of the spatial distribution within the pools, coliforms continue to persist in some pool environments. The persistence of total coliforms in Lechuguilla Cave after a one-year closure indicates there may be an energy source within the pools supporting sustained coliform presence. The persistence is hard to explain otherwise.

BIOFILM ENHANCEMENT OF COLIFORM GROWTH

Public understanding of microbiology, the use of indicator organisms and the risks to human health from microorganisms is often poor (Shagam *et al.* 2000). Microbes maintain nutrient cycles, decompose organic material, and support the growth of plants and animals. Certain microbes like coliforms, however, can be indicators of microbes harmful to human health, especially where microbial numbers are enhanced by anthropogenic activities.

Why are coliforms surviving in an environment such as Red Lake that has been closed to human access? What energy sources are they using to replace those obtained in the human digestive tract? Previous research has shown carbon compounds are secreted from hose materials which can provide nutrients for the survival of select microbes (Budde 1995). Additional research indicates soil material at Huapache camp in the Western Branch to be rich in clay minerals (Spilde, personal communication in 1999). Clay is known to protect microorganisms and organic compounds from environmental factors (Van Veen and Kuikman 1990, Scott *et al.* 1996, Hassink 1997, Vettori *et al.* 2000). Therefore, the possibility exists that coliforms may reside in clay-rich soils and may be transported via cavers' boots to other soils and water sources within the cave. Secondly, normal underground living conditions often results in cavers leaving behind organic substances such as fecal matter, hairs, skin particles, fingernails, sputum, food constituents and lint from clothing that could provide organics which might support coliform survival.

The growth of coliforms is also enhanced in the presence of biofilms and cultures of *Hyphomicrobium*-like organisms. Results from laboratory experiments indicate that the heaviest coliform growth occurred on diluted cave water samples containing biofilm. Cave pools with siphoning hoses that support biofilm growth thus appear to be supplying additional carbon and nutrients to the water and may contribute to the persistence of coliforms within the biofilms. When a growth medium (e.g. cave water) is rich in nutrients, bacteria will attach randomly to any available surface; however, bacteria will preferentially attach to a nutritive surface in oligotrophic, nutrient-poor conditions such as those in Lechuguilla pools (Watnick and Kolter 2000). Therefore, introducing a leachable, energy-rich hose into an oligotrophic environment will cause native bacteria to accumulate on the hose surface and possibly increase the persistence of introduced organisms such as coliforms. Hoses in Lechuguilla Cave are currently increasing the numbers of both native bacteria and introduced coliforms. However, by replacing the tubing with a more inert thermoplastic that reduces or eliminates biofilm growth, we hypothesize that the coliforms will no longer have a nutrient and energy source and will not persist.

BIOFILM GROWTH ON TUBING

VWR Tygon is the tubing currently used in Lechuguilla's drinking-water resources. Unfortunately, this tubing also promoted the heaviest biofilm growth of the six hose materials tested. VWR Tygon contains polyvinylchloride (PVC), polyurethane, and phthalates, which are added to make tubing soft and bendable. Plasticizers also aid in the flexibility of these tubing types. The oily, organic-rich compounds within plasticizers support bacterial and fungal growth. Tubing promoting moderate growth after five months also contained several additives that could have supported biofilm growth. Lipids and fatty acids, for instance, are present in natural rubber latex, which can leach when tubing is left in water for long periods of time (Beacon Pharmaceuticals 2001). Nalgene Premium Tygon 180 also contains PVC and plasticizers that might support microbial growth (Cole-Parmer 1998). Although Norton Tygon did not promote biofilm growth, we are not recommending use of this hose for siphoning water in cave pools. Norton Tygon tubing contains PVC resin and can emit plasticizers that could enhance microbial growth over longer periods of time than were tested in this study or even present a toxicity risk to native microorganisms.

Because cave water was used and the tubing was left to grow in a dark Lechuguilla-temperature laboratory incubator, similar results can be expected in Lechuguilla cave pools. The tubing results might also be applicable to other buffered, oligotrophic water conditions outside cave environments such as distilled water lines in biological/chemical laboratories, medical and dental hoses, and possibly food and beverage dispensers where biofilm communities are a problem (Tall *et al.* 1995).

Silicone Masterflex and Teflon FEP (fluorinated ethylene propylene) tubing did not support microbial growth in lab experiments and would be good alternatives for siphoning hoses within Lechuguilla pools. These hoses do not contain plasticizers and each has fungus-resistant properties, preventing fungal growth on the inside and outside of the tubing. Silicone platinum-cured tubing contains water-based materials and is composed of siloxane polymers and amorphous silica, non-nutritive substances. Additionally, the platinum curing provides a lower level of protein binding with fewer potential leachable compounds. Teflon is the most resistant fluorocar-

bon and the most chemically inert thermoplastic known. It is very stiff tubing, and the added FEP used in manufacturing discourages microbial growth (Cole-Parmer 1998).

RECOMMENDATIONS

We suggest several changes in current practices to correct human-associated microbial contamination problems at water sources and camp/urine locations in Lechuguilla Cave. Ultimately, these recommendations should help resolve the persistence of coliforms and other non-native bacteria in cave pools. Past remediation techniques in the Red Lake area have included using small amounts of hydrogen peroxide on flowstone where coliforms are suspected and scrubbing to remove boot marks (Boston 1999). Recommendations to eliminate food caching, circumventing the need to swim pools to cross them, and eliminating bivouacking in random areas have been noted in past documents as ways to reduce contamination (Northup *et al.* 1992). Limiting travel in the cave to essential surveying, exploration, and scientific trips, and encouraging cavers to have clean clothes, hair and boots when entering the cave also have been suggestions to help reduce the amount of organic carbon and number of new microorganisms introduced into Lechuguilla Cave (Northup *et al.* 1992).

Current recommendations stemming from this research include routine use of positive/negative total coliform indicator test kits at all water locations. This will help Carlsbad Caverns National Park Service personnel to monitor human impact at pool locations. Human impact can be further reduced by using boot covers at urine dumps and drinking-water sites, sterile gloves (especially when dipping water pitchers into pools), and clean Tyvek suits when approaching more pristine drinking-water pools (e.g. Red Lake, Deep Secrets Pool, Oasis, Pearlsian Gulf, Big Sky Pool). Packing out all fecal and urine waste in properly sealed containers would also help reduce new sources of contamination. Removal of the current VWR Tygon tubing from the pools and insertion of silicone Masterflex (Cole-Parmer catalog # 96410-15) or Teflon-FEP tubing (Cole-Parmer catalog # P-06406-12) should keep biofilm populations from proliferating on drinking-water hoses. Another consideration might include replacing hose spigots with material similar in composition to the recommended hoses (silicone or Teflon). Additionally, iodine tablets or a water purification system should be used when drinking from contaminated pools where coliform numbers are above 0 organisms/100mL. These precautionary measures will ultimately reduce human impact and hopefully eliminate or reduce human-associated bacteria from water sources and other pristine locations over time.

CONCLUSIONS

Coliform presence is currently a problem in water sources within Lechuguilla Cave. Most tested pools contain coliforms in numbers that are unacceptable under the Environmental Protection Agencies limits for potable water. Remediation techniques and area exclusion seem to have had limited success in efforts to eliminate these organisms. Coliform persistence in some water sources continues. Pools with siphoning hoses appear to offer coliforms an additional organic food source in the form of biofilms residing on tubing used for water collection. Lab experiments show coliform growth to be enhanced in the presence of biofilm, preventing the die-out of coliforms in pools with siphoning hoses. Using an alternative siphoning hose such as silicone or Teflon that have limited, leachable organics and lack plasticizers will reduce biofilm growth.

Adding new materials such as siphoning hoses in a pristine environment can be risky business if they have not been previously tested. Consequences range from disturbing native microflora and enhancing non-native human-introduced organism populations to introducing biological and chemical pollutants into a habitat that has remained secluded from surface interference for long periods of time. It is important to take precautions when adding anthropogenic materials to pristine environments. Reducing human impacts and contamination to cave soils and water sources is essential both for human health and for answering scientific questions regarding natural microbial communities within deep karstic environments.

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REFERENCES

American Public Health Association, 1989, Standard methods for the examination of water and wastewater 20th Edition: New York, American Public Health Association, 1,220 p.

Atlas, R.M., 1995, Handbook of media for environmental microbiology: Boca Raton, CRC Press, 222 p.

Beacon Pharmaceuticals, 2001, Latex allergy information pack, http://www.aimultimedia.com/aidsmythexposed/pdf/beacon_allergy.pdf, p. 1-32.

Boston, P.J., 1999, Red lake contamination. Unpublished technical report to Carlsbad Caverns National Park, Carlsbad, New Mexico, 6 p.

Boston, P.J., 2000, Life below and life 'out there:' Geotimes, v. 45, p. 14-17.

Boston, P.J., Spilde, M.N., Northup, D.E., Melim, L.A., Soroka, D.S., Kleina, L. G., Lavoie, K.H., Hose, L.D., Mallory, L.M., Dahm, C.N., Crossey, L.J., & Schelble, R.T., 2001, Cave biosignature suites: Microbes, minerals and Mars. Astrobiology Journal, v. 1(1), p. 25-55.

Budde, K, 1995, Outgassing of materials, *in* Materials Research Society Proceedings, v. 386, p. 165-169.

Chapra, S.C., 1996, Water resource handbook: New York, McGraw Hill, p. 1011.

Cole-Parmer, 1998, Cole-Parmer instrument company catalog 1999-2000 version: Vernon Hills, Cole-Parmer Instrument Catalog, 1141 p.

Cunningham, K.I., Northup, D.E., Pollastro, R.M., Wright, W.G., and LaRock, E.J., 1995, Bacteria, fungi and biokarst in Lechuguilla Cave, Carlsbad Caverns National Park, New Mexico: Environmental Geology, v. 25, p. 2-8.

Dawson, H.E., 1996, Hydrochemistry of Lechuguilla Cave. Unpublished technical report to Carlsbad Caverns National Park, Carlsbad, New Mexico, 6 p.

De Bont, J.A.M., Van Dijken, J.P., and Harder, W., 1981, Dimethyl sulphoxide and dimethyl sulphide as a carbon, sulphur, and energy source for growth of *Hyphomicrobium*: Journal of General Microbiology, v. 127, p. 315-323.

Environmental Protection Agency, 1999, National primary drinking-water standards: Office of Water, http://www.epa/gov/safewater/hfacts/html.

Hassink, J., 1997, The capacity of soils to preserve organic C and N by their association with clay and silt particles: Plant and Soil, v. 191, p. 77-87.

Hill, C.A., 2000, Overview of the geologic history of cave development in the Guadalupe Mountains, New Mexico: Journal of Cave and Karst Studies, v. 62, p. 60-71.

Hirsch, P., and S. F. Conti, 1964a, Biology of budding bacteria. I. Enrichment, isolation and morphology of *Hyphomicrobium* spp: Archives of Microbiology, v. 48, p. 339-357.

Hirsch, P., and S. F. Conti, 1964b, Biology of budding bacteria. II. Growth and nutrition of *Hyphomicrobium* spp: Archives of Microbiology, v. 48, p. 358-367.

Koch, A.L., 1994, Growth measurement, in Gerhardt *et al*., eds. Methods for general and molecular bacteriology: Washington, DC, American Society for Microbiology, p. 248-277.

Lappin-Scott, H., and Costerton, J.W., 1997, McGraw Hill Encyclopedia of Science and Technology, 8th ed: New York, McGraw Hill, p. 662-663.

Mallory, L., Spokas, L., and Northup, D., 1995, Numerical taxonomic comparison of two freshwater microbial communities in Lechuguilla Cave, Carlsbad Caverns National Park: General Meeting of the American Society of Microbiology Abstracts, v. 95, no. 0, p. 442.

Myers, D.N., and Sylvester, M.A, 1998, Fecal indicator bacteria, *in* Wilde, F.D., Radtke, D.B., Gibs, J., Iwatsubo, R.T., eds. National field manual for the collection of water-quality data, TWRI Book 9: Reston, U.S. Geological Survey, p. 1-38.

Northup, D.E., Carr, D.L., Crocker, M.T., Cunningham, K.I., Hawkins, L.K., Leonard, P., & Welbourn, W.C., 1992, Lechuguilla cave biological inventory. Unpublished technical report to Carlsbad Caverns National Park, p. 175.

Northup, D.E., Beck, K.M., & Mallory, L.M, 1997, Human impact on the microbial communities of Lechuguilla cave: is protection possible during active exploration?: Journal of Cave and Karst Studies, v. 59, p. 166.

Northup, D.E., Dahm, C.N., Melim, L.H., Spilde, M.N., Crossey, L.J., Lavoie, K.H., Mallory, L.M., Boston, P.J., Cunningham, K.I., & Barns, S.M., 2000, Evidence for geomicrobiological interactions in Guadalupe caves: Journal of Cave and Karst Studies, v. 62, p. 80-90.

Palmer, A.N., Palmer, M.V., 2000, Hydrochemical interpretation of cave patterns in the Guadalupe mountains, NM: Journal of Cave and Karst Studies, v. 62, p. 91-108.

Poindexter, J.S., 1992, Dimorphic prosthecate bacteria: The genera *Caulobacter*, *Asticcacaulis*, *Hyphomicrobium*, *Pedomicrobium*, *Hyphomonas*, and *Thiodendron*, *in* Balows, A., ed. The prokaryotes-a handbook on the biology of bacteria: Ecophysiology, isolation, identification, applications: NewYork, Springer-Verlag, p. 2176-2192.

Polyak, V.J., & Provencio, P.P., 2000, Summary of the timing of sulfuric acid speleogenesis for the Guadalupe caves based on ages of alunite: Journal of Cave and Karst Studies, v. 62, p. 72-74.

Provencio, P.P., & Polyak, V.J. 2001, Iron oxide-rich filaments: Possible fossil bacteria in Lechuguilla Cave, New Mexico: Geomicrobiology Journal, v.18, p. 297-309.

Sambrook, J., Fritsch, E.F., & Maniatis, T., 1989, Molecular cloning, a laboratory manual 2nd edition: New York, Cold Spring Harbor Laboratory Press, 3 vols.

Scott, N., Cole, C., Elliott, E.T., and Huffman, S., 1996, Soil textural control on decomposition and soil organic matter dynamics: Soil Science Society of America Journal, v. 60, p. 1102-1109.

Shagam, J.Y., French, R., & Hogrefe, R., 2000, Public health study and assessment of middle Rio Grande and Albuquerque's reclaimed water-wastewater master plan for 2000-2010, in New Mexico Environmental Health Conference 2000, 110 p.

Sperl, G.T., & Hoare, D.S., 1971, Denitrification with methanol: A selective enrichment for *Hyphomicrobium* species: Journal of Bacteriology, v. 108(2), p. 733–736.

State of New Mexico, 1995, Standards for interstate and intrastate streams: State Records Center Open-File Report 20 NMAC 6.1.

Suylen, G.M.H., Stefess, G.C., Kuenen, J.G., 1986, Chemolithotrophic potential of a *Hyphomicrobium* species, capable of growth on methylated sulphur compounds: Archives of Microbiology, v. 146, p. 192-198.

Tall, B.D., Williams, H.N., George, K.S., Gray, R.T., and Walch, M., 1995, Bacterial succession within a biofilm in water supply lines of dental air-water syringes: Canadian Journal of Microbiology, v. 41, p. 647-654.

Todar, K., 2002, Pathogenic *E.coli*: Todars Online Textbook of Bacteriology, http://textbookofbacteriology.net/e.coli.html.

Turin, H.J., & Plummer, M.A., 2000, Lechuguilla cave pool chemistry, 1986-1999: Journal of Cave and Karst Studies, v. 62, p. 135-143.

Van Veen, J.A., & Kuikman, P.J., 1990, Soil structural aspects of decomposition of organic matter by micro-organisms: Biogeochemistry, v. 11, p. 213-233.

Vettori, C., Gallori, E., & Stotzky, G., 2000, Clay minerals protect bacteriophage PBS1 of *Bacillus subtilis* against inactivation and loss of transducing ability by UV radiation: Canadian Journal of Microbiology, v. 46(8), p. 770-773.

Watnick, P., & Kolter, R., 2000, Biofilm-city of microbes: Journal of Bacteriology, v. 182, p. 2675-2679.

Webb, W.E., Radtke, D.B., & Iwatsubo, R.T., 1998, Surface-water sampling: Collection methods at flowing-water and still-water sites, *in* Wilde, F.D., Radtke, D.B., Gibs, J., Iwatsubo, R.T., eds., National field manual for the collection of water-quality data TWRI Book 9: Reston, U.S. Geological Survey, p. 1-38.

Whittenbury, R., Phillips, K.C., and J. F. Wilkinson, 1970, Enrichment, isolation and some properties of methane-utilizing bacteria: Journal of General Microbiology, v. 61, p. 205-218.